Comparative Thermodynamic Linkage Study of the Calcium-Induced Solubility of Bovine and Caprine Caseins

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In this paper, the calcium-induced solubility profiles of isolated caprine α_{s1}-casein and both bovine and caprine whole caseins, containing known amounts of α_{s1}, have been analyzed and compared using Wyman's theory of thermodynamic linkage in conjunction with nonlinear regression analysis. All of the solubility profiles could be described by an initial salting-out followed by salting-in. These events were quantified by a salting-out constant, k_1, and a salting-in constant, k_2. These constants are correlated with association constants for salt binding so that n and m, the number of moles of calcium bound to different classes of sites which induce the respective changes in solubility, could also be quantitated. The purified α_{s1}-casein from caprine milk differs from its bovine counterparts in that the parameters k_1 and n are somewhat smaller, indicating less salt binding at both 24 and 1 °C. Conversely, for whole caseins, k_1 was 6 times larger for caprine, which contains significantly less α_{s1}-casein, than for bovine whole casein. This suggests stronger Ca^{2+}–casein interactions in caprine whole casein as compared to whole bovine casein. Binding of Ca^{2+} ions to stronger affinity sites results in greater "salting-out" for caprine whole casein. Studies on temperature and ionic strength dependence of the comparative solubilities confirm this hypothesis.

INTRODUCTION

Casein occurs in milk as nearly spherical colloidal particles. These particles, the so-called micelles, consist of a large number of phosphorylated protein subunits as well as inorganic calcium and phosphate. Casein is not a single protein, but in ruminant milk consists of four main components, α_{s1}-, α_{s2}-, β-, and κ-casein, which self-associate to form limiting polymers termed submicelles (Schmidt, 1982; Farrell and Thompson, 1988). Presumably, the submicelles are in turn bound together to form the casein micelles by calcium salt bridges with carboxylic and ester phosphate groups of the protein moiety (Schmidt and Payens, 1976).

α_{s1}-, α_{s2}-, and β-casein are readily precipitated by calcium ions, whereas κ-casein is not. On the contrary, the latter component is able to stabilize the other casein components against the flocculating action of calcium ions by the formation of stable colloidal micelles at physiological conditions. However, in model systems, as the concentration of calcium is increased to 50 mM, destabilization again occurs (Kumosinski and Farrell, 1991). Thus, calcium ions exert a number of important effects on casein solubility in solutions. In synthetic casein micelles, variation of the contents of three of the four major caseins (α_{s1}, β, and κ) can produce varying size distributions of casein micelles (Schmidt et al., 1977) which in turn have very different functional properties (Schmidt and Koops, 1977; Kumosinski and Farrell, 1991).

Previous research on caprine caseins has shown that a distribution of the quantity of α_{s1}-casein occurs among individual animals (Grosclaude et al., 1987; Ambrosoli et al., 1988; Brignon et al., 1989). In caprine milks, β-casein is the predominant casein, while the amounts of α_{s1} appear to vary genetically from animal to animal, ranging from none to 30% that of bovine (Mora-Gutierrez et al., 1991). The caprine system offers a unique opportunity to test the effects of quantitative changes in casein components on variation of functional properties.

One functional property has been studied in detail—solubility in solutions of calcium; changes induced in solubility by the binding of calcium to the individual purified bovine caseins and to casein micelles have been quantified (Farrell and Kumosinski, 1988; Farrell et al., 1988; Kumosinski and Farrell, 1991). Analyses of such solubility profiles were accomplished using Wyman's theory of thermodynamic linkage (Wyman, 1964). To test the effects of variation in α_{s1}-casein and to further characterize the calcium-induced solubility changes in caseins other than bovine, we have quantified the binding of calcium to whole caprine casein and its α_{s1}-component through the use of the same approach. The solubility behavior of whole bovine casein was also studied for comparison.

MATERIALS AND METHODS

Deionized Water. Deionized water, prepared by passage of distilled water over a mixed bed cation–anion exchanger, was used throughout this study.

Sources of Caseins. Whole caseins were prepared by isoelectric precipitation of the individual skim milk samples obtained from a Jersey cow and a French Alpine goat. The precipitate was dissolved by addition of NaOH to yield a solution of pH 7.0. The casein was reprecipitated, washed, and then resuspended. The sodium caseinate was subsequently cooled to 4 °C and centrifuged at 100000g for 30 min to remove residual fat. Finally, the suspension was dialyzed against cold deionized water at 4 °C for 72 h with three changes and then lyophilized.

Purified caprine α_{s1}-casein was obtained as described: crude α_{s1}-casein was prepared by urea fractionation (Thompson, 1966) followed by column chromatography on DEAE-cellulose in urea (Thompson and Kiddy, 1964).

The integrity of the samples was confirmed by SDS–PAGE and the percentages of the various component caseins estimated by densitometry as previously described (Basch et al., 1989).

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The α_{s1}-casein content of the caprine whole casein was estimated to be 2.70 g/L by RP-HPLC (Mora-Gutierrez et al., 1991).

Solubility of Caseins. Solubility of caseins at each of two temperatures (1 and 24 °C) was carried out as follows:

(1) Dissolve caseins (about 20 mg/mL) in water and adjust pH to 7.0 with 0.1 N KOH or NaOH. Equilibrate in water bath at desired temperature for 15–20 min.

(2) To 2 mL of protein solution (in thick-walled centrifuge tubes), add 2 mL of CaCl$_2$ solutions, with or without buffer ± KCl. Invert the tube and let stand at desired temperature for 30 min.

(3) Centrifuge for 15 min at 43654g_{max} at desired temperature in a Beckman Model L-7 ultracentrifuge with an SW 60 Ti swinging bucket rotor.

(4) Transfer 1 mL of supernatant to a 10-ml volumetric flask containing 1 mL of 1 N sodium citrate plus a few milliliters of water; make up to volume with water. When solubility is determined at 1 °C, pipets must be prechilled to avoid precipitation of protein in the pipet. Read in 1-cm cuvettes at 280 nm. An absorptivity of 0.850 mL/mg at 280 nm was used for whole casein. The $A_{280}^{1\%}$ for α_{s1}-casein was taken as 10.0 (Thompson and Kiddy, 1964).

Theory and Data Analysis. Wyman's (1964) concept for linked functions was shown to be useful for the treatment of the calcium-induced sequential precipitation (salting-out) and resolubilization (salting-in) of individual bovine casein casein components (Farrell and Kumosinski, 1988; Farrell et al., 1988). The best fit was found for a model assuming two classes of binding sites for the ions responsible for the above-mentioned sequential processes. The equilibria involved were formulated as

$$M + nX \overset{k_1^n}{=} MX_n + mX \overset{k_2^m}{=} MX_nX_m \qquad (1)$$
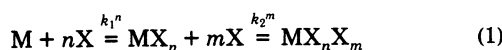
where M is the unbound protein, X is the free calcium, n and m are the number of moles of X bound to the two sites, and k_1 and k_2 are the apparent binding constants per site. The measured quantity was apparent protein solubility S'. Defining S_0, S_1, and S_2 as the intrinsic solubilities of M, MX$_n$, and MX$_n$X$_m$, respectively, the model for S' has the form

$$S' = f_M S_0 + f_{MXn} S_1 + f_{MXnXm} S_2 \qquad (2)$$

By expressing the fractional components in terms of the above equilibria and making the assumption that $X = C_x$ at high relative concentration of salt, the following model was formulated as previously described (Farrell et al., 1988):

$$S' = S_0/(1 + k_1^n C_x^n) + S_1 k_1^n C_x^n/(1 + k_1^n C_x^n) + \\ (S_2 - S_1)k_2^m C_x^m/(1 + k_2^m C_x^m) \qquad (3)$$

This sequential binding model assumes that $k_1 \gg k_2$ and the n sites become saturated with X prior to the occurrence of significant binding of X to the m sites. Also, for n or m greater than one, k_1 and k_2 represent an average value for each class of the n or m binding sites. In reality, n or m moles of salt will bind with only one equilibrium constant (k_1), i.e., $K_1 = k_1^n$ and $K_2 = k_2^m$. Note that C_x in eq 3 represents the total salt concentration. For comparative purposes this represents a practical approach. Since the degree of aggregation of the whole casein is large, the relative concentration of protein is lower and only total calcium is used. When needed, the actual bound/free ratio can readily be calculated as previously described (Kumosinski and Farrell, 1991) and the data reanalyzed as a function of free calcium.

The sequential binding model in eq 3 was applied in the present study to the calcium ion-induced solubility profiles of bovine and caprine whole casein and its α_{s1} component. The salt-induced solubility profiles were analyzed using an iterative nonlinear regression program (NLLSQ in BASIC) on an IBM/PC-AT microcomputer which employed the Marquardt algorithm. This program minimized the standard deviation of the experimental points from the curve, also known as the root mean square deviation (RMSD), where the RMSD is defined as

$$RMSD = SS/(NO - NP + NX) \qquad (4)$$

where SS is the sum of the squares of the differences $(YC - Y)$ between the calculated and observed Y values, NO is the number
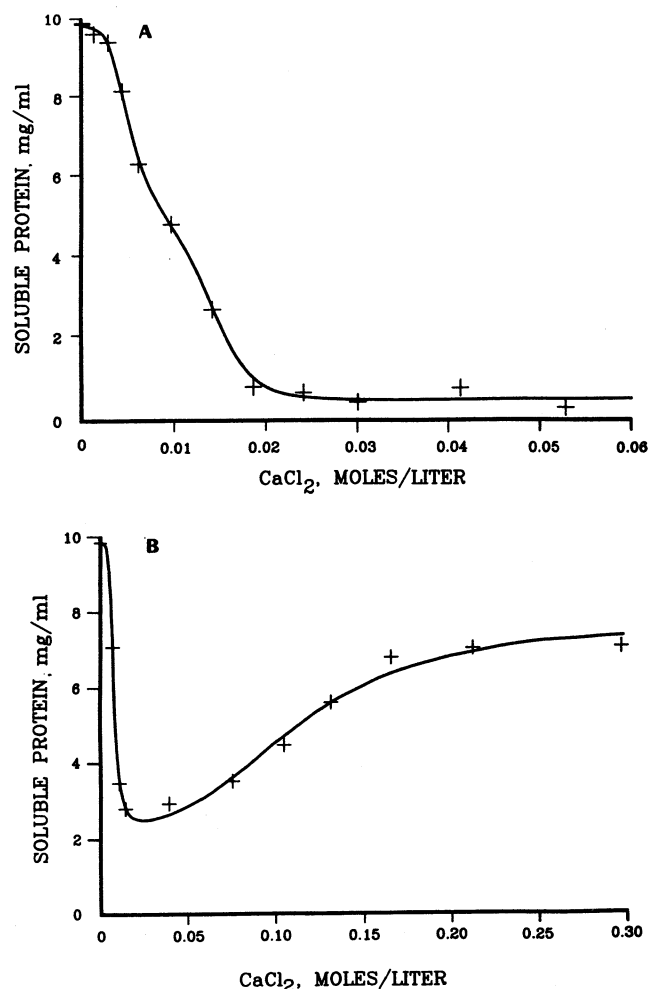
Figure 1. Solubility at (A) 24 °C and at (B) 1 °C of caprine α_{s1}-casein as a function of increasing CaCl$_2$ concentration. Solutions were buffered at pH 7.0, 10 mM imidazole hydrochloride with 0.07 M KCl. The experimental data represent the average of triplicate determinations and were fitted by eq 3 by nonlinear regression analysis; the curves represent an artist's rendition of the computer-generated lines. Results are given in Table I.

of data points, NP is the number of parameters, and NX is the number of excluded parameters.

The n and m values chosen yielded the minimum root mean square deviation value for the analysis with the minimum error in both k_1 and k_2.

Densitometry. Densitometric measurements of the whole caseins were conducted as previously described (Basch et al., 1989). In these studies, however, a Bio-Rad Model 222 gel scanner was used. Data were analyzed using software programs supplied by the manufacturer.

RESULTS AND DISCUSSION

Caprine α_{s1}-Casein. *A. Solubility at 24 and 1 °C.* The amino acid sequence of caprine α_{s1}-casein is nearly identical with that of α_{s1}-casein B, the normally occurring genetic variant in bovine milk (Brignon et al., 1989). The solubility of purified caprine α_{s1}-casein was determined as a function of calcium ion concentration (Figure 1A). As the calcium ion concentration approaches 0.02 M, the protein precipitates and remains insoluble up to 0.1 M CaCl$_2$ (extended data not shown). This behavior is similar to that observed for purified α_{s1}-B from bovine milk (Farrell et al., 1988). At low concentrations the protein is soluble, but as the calcium concentration is increased and calcium ions bind to the phosphoserine residues of the purified casein, charge neutralization occurs, altering the protein net charge and leading to precipitation at even

Table I. Comparison of Calcium-Induced Insolubility and Solubility of Caprine and Bovine α_{s1}-Caseins at 24 and 1 °C[a]

temp, °C	casein	k_1, L/mol	n	k_2, L/mol	m	S_2,[b] g/L
24	caprine α_{s1}	105 ± 9	5			
37	bovine α_{s1}-B[c]	186	8			
	bovine α_{s1}-A[c]	157	8			
1	caprine α_{s1}	127 ± 4	5	9.0 ± 0.7	3	5.1 ± 0.3
	bovine α_{s1}-B[c]	123	8	2.5	4	4.0
	bovine α_{s1}-A[c]	68	8	10.6	8	8.5

[a] Solutions buffered at pH 7.0, 10 mM imidazole hydrochloride, 0.07 M KCl. [b] S_2 denotes the concentration of soluble species after salting-in as defined in eq 1. [c] Farrell et al. (1988).

higher concentrations of calcium. The corresponding number of calcium ions bound was correlated with n of eq 3. The reduction in net charge will allow the proteins to interact to form association complexes, and a decrease in solubility is to be expected. All of the solubility profiles (Farrell et al., 1988) are therefore thermodynamically linked to salting-in and salting-out due to ion binding. The magnitude of the k_1 parameters suggested involvement of protein phosphate groups in binding and precipitation (Farrell et al., 1988) when compared with known values for the association constants. For caprine α_{s1}-casein, the values of k_1 and n obtained by use of eq 3 are given in Table I and are different from those obtained of the bovine protein, although no salting-in occurred for either protein at temperatures ≥24 °C. For caprine α_{s1}, k_1 is in agreement with K_A for Ca^{2+} binding (Dickson and Perkins, 1971) but n = 5 does not approximate the number of phosphate groups on the protein (Brignon et al., 1989). The lower n value for the purified caprine casein could imply a decreased net negative charge or a more concerted mode of binding of Ca^{2+}, with a significantly lower k_1.

Figure 1B shows the solubility of caprine α_{s1}-casein as a function of $CaCl_2$ concentration at 1 °C. Here hydrophobic interactions are minimized (Farrell et al., 1988) so that cation binding and its resultant effects can be highlighted. As a consequence of calcium binding with the negatively charged groups of the protein, charge neutralization occurs and even at 1 °C the solubility decreases as Ca^{2+} concentration increases up to approximately 0.02 M. Solubility then increases to a limiting value. The process was quantified by eq 3 (Table I). The data in Figure 1B were previously explained (Farrell et al., 1988) in terms of two different classes of calcium binding sites. At low concentrations of $CaCl_2$, there is significant binding of Ca^{2+} to the relatively strong sites (phosphate groups); this binding has been correlated with salting-out. Next, binding of Ca^{2+} to weaker sites (carboxylate groups) is thought to occur at higher $CaCl_2$ concentrations. It is the binding to these latter sites that has been correlated with salting-in. At 1 °C, a good correlation was observed between the salting-out constant k_1 of caprine α_{s1}-casein (127 L/mol) and that of the less soluble bovine α_{s1}-casein, the B variant (k_1 = 123 L/mol; Farrell et al., 1988). At 24 °C hydrophobic interactions dominate, and subtle differences can be missed. However, at 1 °C, where hydrophobic interactions are minimized, salt–protein interactions are amplified. Here, the n and m values, namely 5 and 3, were different from the corresponding 8 and 4 for bovine α_{s1}-B casein (Farrell et al., 1988). This implies that the caprine α_{s1}-casein might actually bear lower net negative charge and bind less calcium. At 1 °C, the value of k_2 for caprine α_{s1}-casein also differs significantly from that of the bovine protein (Table I). Additionally, the amount of protein solubilized at 1 °C over the range of $CaCl_2$ tested is also higher for caprine α_{s1}-casein. These results indicate that at 1 °C, although similar in sequence to α_{s1}-B, the caprine α_{s1}-casein behaves in an intermediate fashion between α_{s1}-B
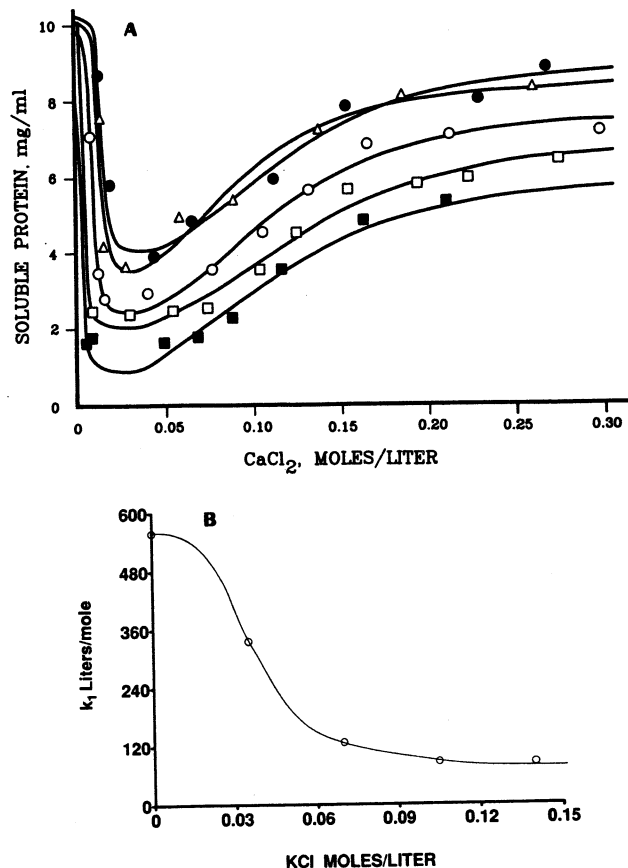

Figure 2. (A) Solubility at 1 °C of caprine α_{s1}-casein as a function of increasing $CaCl_2$ and KCl concentrations. (●) 0.140 M KCl; (▲) 0.105 M KCl; (○) 0.07 M KCl; (□) 0.035 M KCl; (■) no buffer or KCl. Data represent the average of triplicate determinations and were fitted by eq 3; curves are as explained in Figure 1. Results are given in Table II. (B) Replot of values of k_1 from (A) as a function of concentration of KCl. Data were fitted by eq 3 with n = 3.

and α_{s1}-A, a 13-residue deletion mutation of B which is nearly completely salted-in under similar conditions (Table I).

B. Influence of Electrolyte on Salting-out and Salting-in Constants. Figure 2A shows that at 1 °C and pH 7.0 the calcium-induced solubility profile of caprine α_{s1}-casein can be altered by addition of KCl. Increasing ionic strength for each calcium-induced solubility profile of caprine α_{s1}-casein decreased k_1 values (Table II). Figure 2A clearly demonstrates that the interaction between caprine α_{s1}-casein and calcium also depends strongly upon the concentration of KCl. A replot of the k_1 values (Figure 2B) analyzed with eq 3 yielded a k value of 27 M^{-1} for K^+ with n = 3, in agreement with the effect on bovine α_{s1}-A. KCl addition leads to a more effective electrostatic repulsion due to competition of potassium for calcium binding sites located on the surface of the caprine α_{s1}-casein molecule. The effect is not simply due to ionic strength as, for example, increased $CaCl_2$ does not have

Table II. Ionic Strength Dependence of Calcium-Induced Solubility of Caprine α_{s1}-Casein at 1 °C

KCl, mM	k_1, L/mol	k_2, L/mol	S_1,[a] g/L	S_2,[b] g/L
0[c]	558 ± 19	8.4 ± 0.8	1.3 ± 0.2	4.5 ± 0.3
35[c]	336 ± 10	7.8 ± 0.6	2.2 ± 0.2	4.6 ± 0.3
70[c]	127 ± 4	8.9 ± 0.7	2.4 ± 0.2	5.1 ± 0.3
105[c]	86 ± 7	10.9 ± 2.4	3.2 ± 0.6	3.8 ± 0.6
140[c]	84 ± 8	8.5 ± 1.8	3.3 ± 0.4	3.8 ± 0.6

[a] S_1 denotes the minimum value for soluble protein after precipitation as defined in eq 1 but before resolubilization. [b] S_2 denotes maximum value for resolubilized (salted-in) protein as defined in eq 1. [c] $n = 3$, $m = 5$.
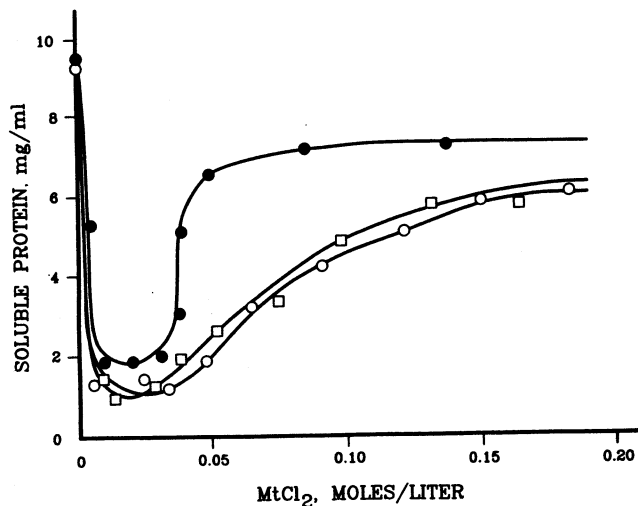

Figure 3. Solubility at 1 °C of caprine α_{s1}-casein as a function of increasing concentration of various salts. (●) MgCl$_2$; (□) CaCl$_2$; (○) CoCl$_2$. Data represent the average of triplicate determinations and were fitted by eq 3; curves are as explained in Figure 1. Results are given in Table III.

nearly the same effect on the no-buffer case (■ of Figure 2A) as do modest KCl increases at constant CaCl$_2$. The values of k_1 are, however, much larger for the caprine α_{s1}-casein than for the bovine α_{s1}-A casein (Farrell et al., 1988). As shown in Table II, the estimated S_1 values, the salting-out solubility, increase with ionic strength, which reflects the considerable electrostatic repulsions as a consequence of the binding K$^+$/Ca^{2+} to the phosphate groups located on the α_{s1}-casein molecule.

By contrast, at high concentrations of Ca^{2+}, this latter cation seems to bind to the protein, with no apparent competition from the K$^+$ ions as shown by the monotonic character of the salting-in, k_2, values (Table II). These values correspond to weak secondary binding sites for calcium binding. This is surprising since a decrease in k_2 upon increasing ionic strength (KCl) is expected. A possible explanation is that only a limited amount of K$^+$ binding can occur in caprine α_{s1}-casein because of internal salt-bridges, and indeed considerably less salting-in occurs as S_2 decreased with KCl (Table II). This hypothesis is consistent with the value of m for caprine α_{s1}-casein being lower than that for the bovine α_{s1}-A casein (8; Farrell et al., 1988), and under comparable conditions bovine α_{s1}-A is completely salted-in at all of the concentrations of KCl used in this study.

C. Effect of Various Cations at 1 °C. Farrell et al. (1988) have evaluated the effects of divalent cations on the solubility of bovine α_{s1}-A casein at 1 °C and pH 7.0. In accordance with our results (Figure 3), the effectiveness of these divalent cations in decreasing the solubility of caprine α_{s1}-casein followd the same order: Co^{2+} > Ca^{2+} > Mg^{2+}. It appears that both α_{s1}-caseins respond to the precipitant action of divalent cations in the same fashion.

Table III. Cation-Induced Solubility of Caprine α_{s1}-Casein at 1 °C

cation	k_1, L/mol	n	k_2, L/mol	m	S_2,[b] g/L
Mg^{2+}	343 ± 26	4	26 ± 1	8	5.6 ± 0.5
Ca^{2+}	245 ± 24	4	15 ± 1	3	5.1 ± 0.2
Co^{2+}	415 ± 152	4	13 ± 1	3	5.2 ± 0.2

[a] S_2 = concentration of soluble caprine α_{s1}-casein as defined in eq 1.

Table IV. Comparison of Casein Distribution of Bovine and Caprine Caseins by Densitometry

casein type	% bovine	% caprine
α_{s2}-casein	10.0	6.2
α_{s1}-casein	38.0	17.1
β-casein	40.0	50.1
κ-casein	12.0	17.5

However, the apparent higher affinities for cation binding (as quantified by k_1 of Table III) make the caprine α_{s1}-casein more sensitive to cation precipitation than either of its bovine counterparts, α_{s1}-A and B. This could be the consequence of a more concerted action of Ca^{2+} binding as n is lower for caprine α_{s1}-casein. As noted above, the degree of resolubilization (S_2) for the caprine casein is intermediate between those for the two bovine genetic variants. The results thus far indicate that whole caprine caseins containing α_{s1} ought to be very different in properties from their bovine counterparts. In addition, since β-casein is the predominant species in caprine milk, the cold solubilities of caprine milk ought to be significantly different from those of whole bovine caseins containing α_{s1}-B.

Bovine and Caprine Whole Casein. *A. Solubility at 24 °C.* As noted above, the calcium-induced solubility changes of individual purified bovine caseins (α_{s1}-A, α_{s1}-B, and β-C casein) were previously thermodynamically linked with their salt-binding capacity (Farrell and Kumosinski, 1988; Farrell et al., 1988). The differences observed for the purified caprine α_{s1}-casein prompted our study of the solubility data for whole bovine and caprine caseins. On the basis of previous studies (Mora-Gutierrez et al., 1991), the milk of an alpine goat rich in α_{s1}-casein was selected for comparison with bovine caseins. The densitometric analysis of their SDS–PAGE profiles is given in Table IV. From these data, it can be seen that the whole bovine casein contains an average distribution of caseins similar to those reported by Davies and Law (1980), while the caprine casein contains significantly less α_{s1}-casein. The solubility properties of the two whole caseins were then compared. Curve A of Figure 4 is for bovine casein, while curve B is for caprine casein.

The salting-out process for purified caseins was quantified by a constant, k_1. As calcium is added to whole casein, synthetic micelles form; then as the calcium content increases, precipitation occurs (Figure 4, curves A and B represent bovine and caprine, respectively). The solubility profiles generated in the present studies of whole casein thus reflect a summation of micelle formation, followed by precipitation. What also occurs for whole casein, which was not apparent in recombined mixtures of purified caseins (Kumosinski and Farrell, 1991), is that when the calcium concentration in a calcium caseinate solution increases, the proteins apparently bind even more calcium, become more positively charged, and have increased solubility at higher concentrations (Figure 4). This salting-in process is quantified by a constant k_2, which can be interpreted as calcium binding to weaker sites (perhaps carboxylate groups). The resulting values pertaining to 24 °C, pH 7.0, in imidazole hydrochloride buffer and 0.07
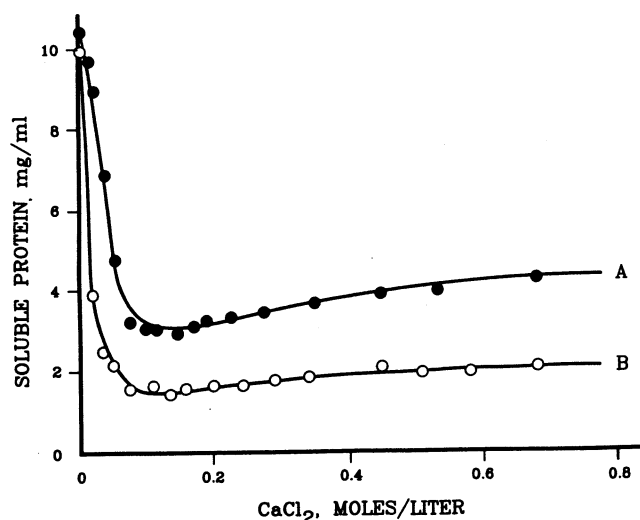
Figure 4. Solubility at 24 °C of bovine (A) and caprine (B) whole casein as a function of increasing $CaCl_2$ concentrations. Solutions buffered at pH 7.0, 10 mM imidazole hydrochloride with 0.07 M KCl. The experimental data represent the average of triplicate determinations and were fitted by eq 3 by nonlinear regression analysis; curves are as explained in Figure 1. Results of analyses are given in Table V.

Figure 5. Solubility at 1 °C of bovine and (A) and caprine (B) whole casein as a function of increasing $CaCl_2$ concentrations. Solutions were buffered at pH 7.0, 10 mM imidazole hydrochloride with 0.07 M KCl. Data in triplicate were fitted by eq 3; curves are as explained in Figure 1. Results of analyses are given in Table V.

M KCl for bovine and caprine whole casein are listed in Table V. The numbers of moles of calcium bound to the different classes of binding sites which leads to solubility changes, n and m, are also listed. Note that here these are small numbers reflecting concerted cooperative interactions in whole caseins.

Comparison of the calcium-induced solubility profiles of bovine and caprine whole casein (Figures 4, A and B, respectively) indicates that at 24 °C whole caprine casein is less soluble than whole bovine casein. Here k_1 was larger than that found for the whole bovine casein (Table V), indicating stronger Ca^{2+}-casein interactions although the degree of salting-out (S_1) was similar. Note also that S_2 of the predicted soluble species is greater for bovine casein at 24 °C. Moreover, small differences in the values of k_2, the salting-in association constant, are observable for both whole caseins (Table V); the small k_2 values could be interpreted as a lower affinity of the carboxyl groups for calcium binding. The lower salting-in effect is, thus, at the root of the overall lower solubility of whole caprine casein.

B. Solubility at 1 °C. The solubilities at 1 °C of bovine and caprine whole caseins as a function of $CaCl_2$ concentration are compared in Figure 5, where curve A represents bovine and curve B caprine. At 1 °C, where hydrophobic interactions are minimized, bovine and caprine whole caseins were more soluble than at 24 °C in the presence of calcium, but the order of solubility remained the same: bovine whole casein > caprine whole casein. From the solubility vs calcium concentration data of Figure 5, and the nonlinear regression analysis by eq 3 (Table V), it is evident that the differences observed are in the calcium association constant k_1. Thus, the lack of solubility behavior of caprine whole casein at 1 °C again might be
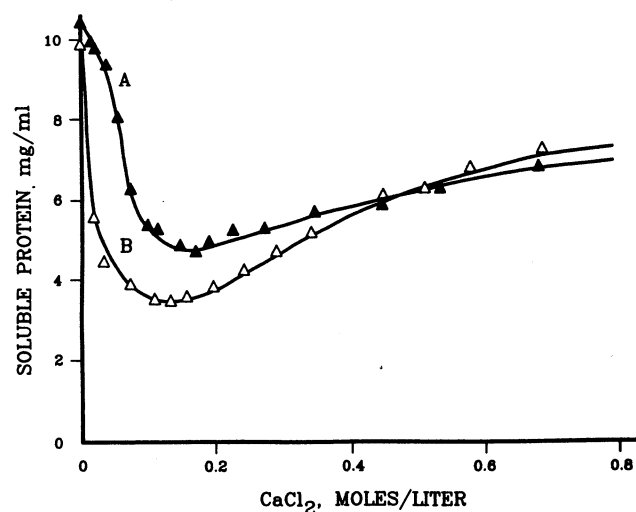
attributable to the strong concerted interaction of calcium with sites on the whole casein. These groups having higher calcium-binding affinities than those of bovine whole casein. Moreover, it should be pointed out that while bovine α_{s1}- and β-caseins alone precipitate at relatively large k_1 values at 24 °C (Farrell and Kumosinski, 1988; Farrell et al., 1988), bovine β-casein is nearly completely soluble under these conditions. In addition caprine α_{s1} is more cold soluble (Table I). Thus, an expected difference in solubility at 1 °C for β-casein-rich caprine casein does not occur. The tighter binding of calcium to caprine phosphorylated serine residues through a cooperative mechanism might account for the differences in solubility illustrated in Figure 5. In addition, the lower values for n for whole caprine caseins (Table V) as compared to bovine caseins may also reflect cooperative interactions over a number of similar sites.

C. Influence of Electrolyte on Salting-out and Salting-in Constants. The effect of varying concentration of KCl on the calcium-induced solubility of whole bovine and caprine caseins at 1 °C is shown in Figure 6, parts A and B, respectively. Here hydrophobic interactions are minimized, and the effect of KCl on the strength of ionic interactions in the precipitate can be tested through the thermodynamic linkage equations. The data were analyzed and fitted by eq 3; parameters k_1, k_2, S_1, S_2, n, and m are given in Tables VI and VII. Our data exhibited behavior similar to that of purified bovine α_{s1}-A casein with increasing solubility in $CaCl_2$ and KCl concentrations (Farrell et al., 1988); this is quite different from the behavior exhibited by synthetic mixtures of α_{s1}-B and κ-casein (Kumosinski and Farrell, 1991) and from the behavior expected for the β-casein-rich whole caprine casein. Increasing ionic strength by the addition of KCl for each calcium-induced solubility profile of bovine and

Table V. Calcium-Induced Insolubility and Solubility of Whole Caseins at 24 and 1 °C[a]

whole casein	k_1, L/mol	n	S_1,[b] g/L	k_2, L/mol	m	S_2,[c] g/L
bovine, 24 °C	24 ± 0.8	3	1.1 ± 0.1	6.9 ± 0.7	2	3.3 ± 0.2
caprine, 24 °C	137 ± 13	1	0.8 ± 0.2	4.4 ± 0.6	2	1.2 ± 0.3
bovine, 1 °C	15 ± 0.9	3	1.0 ± 0.1	5.3 ± 0.8	1	6.4 ± 0.6
caprine, 1 °C	114 ± 9	1	2.9 ± 0.1	2.5 ± 0.1	3	5.2 ± 0.4

[a] Solutions buffered at pH 7.0, 10 mM imidazole hydrochloride, 0.07 M KCl. [b] S_1 denotes the maximum soluble species after precipitation defined in eq 1. [c] S_2 denotes the soluble protein species after salting-in defined in eq 1.
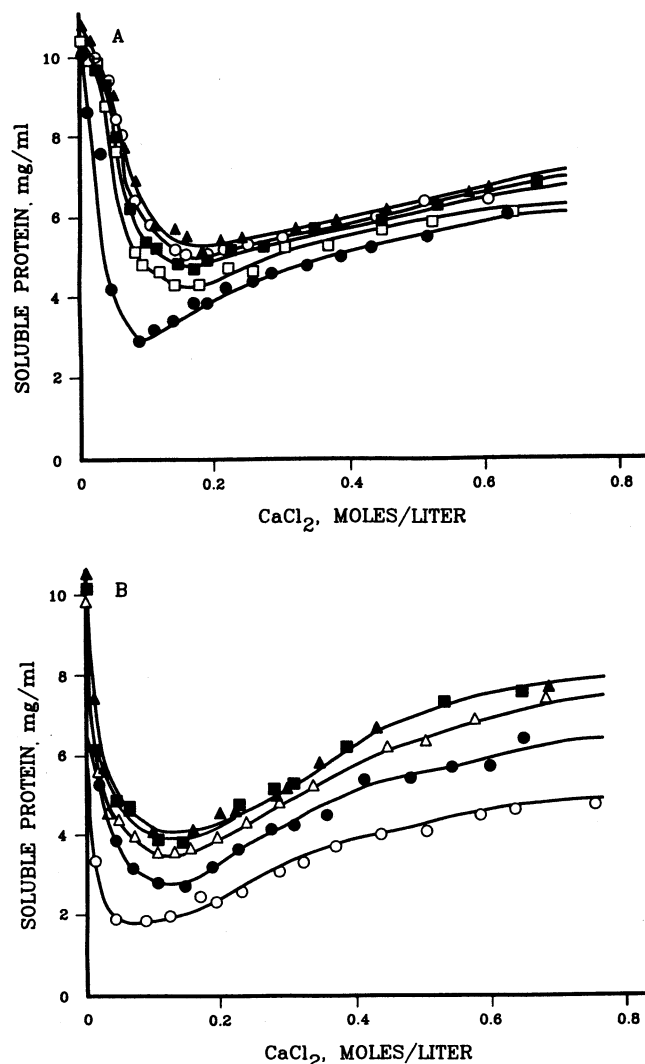
Figure 6. (A) Solubility at 1 °C of bovine whole casein as a function of increasing $CaCl_2$ and KCl concentrations. (▲) 0.140 M KCl; (○) 0.105 M KCl; (■) 0.07 M KCl; (□) 0.035 M KCl; (●) no buffer or KCl. (B) Solubility at 1 °C of caprine whole casein as a function of increasing $CaCl_2$ and KCl concentrations. (■) 0.140 M KCl; (▲) 0.105 M KCl; (△) 0.07 M KCl; (●) 0.035 M KCl; (○) no buffer or KCl. Data in triplicate were fitted by eq 3; curves are as explained in Figure 1. Results are given in Tables VI and VII.

Table VI. Ionic Strength Dependence of Calcium-Induced Solubility of Bovine Whole Casein at 1 °C

KCl, mM	k_1, L/mol	k_2, L/mol	S_1,[a] g/L	S_2,[b] g/L
0[c]	28 ± 2.3	5.6 ± 1.9	1.0 ± 0.1	5.6 ± 1.2
35[d]	18 ± 0.7	3.1 ± 0.8	3.7 ± 0.4	2.9 ± 0.9
70[e]	15 ± 0.9	5.3 ± 0.8	1.0 ± 0.1	6.4 ± 0.6
105[f]	15 ± 0.7	2.6 ± 0.6	4.4 ± 0.5	2.8 ± 0.9
140[g]	15 ± 0.6	2.6 ± 0.7	4.4 ± 0.6	3.0 ± 0.9

[a] S_1, as defined in eq 1, denotes the maximum value for soluble protein after precipitation but before total resolubilization. [b] S_2 denotes the soluble species after salting-in as defined in eq 1. [c] n = 3, m = 1. [d] n = 3, m = 2. [e] n = 3, m = 1. [f] n = 3, m = 2. [g] n = 3, m = 2.

caprine whole casein at 1 °C decreased k_1 and k_2 values from the "no-salt" experiment, but had overall little influence on solubility. Note that purified β-casein is soluble under all of these conditions (Farrell et al., 1988). For purified bovine α_{s1}-casein A a change in the "apparent" association constants for calcium with increasing KCl at 1 °C has been previously ascribed to competition of K$^+$ with Ca^{2+} for charged groups located on the bovine α_{s1}-A casein molecule (Farrell et al., 1988). In the latter studies

Table VII. Ionic Strength Dependence of Calcium-Induced Solubility of Caprine Whole Casein at 1 °C

KCl, mM	k_1, L/mol	k_2, L/mol	S_1,[a] g/L	S_2,[b] g/L
0[c]	276 ± 28	3.2 ± 0.3	1.1 ± 0.2	4.1 ± 0.5
35[d]	87 ± 9	3.5 ± 0.1	1.8 ± 0.2	4.0 ± 0.4
70[e]	114 ± 9	2.5 ± 0.1	2.9 ± 0.1	5.2 ± 0.4
105[f]	78 ± 7	2.7 ± 0.1	3.5 ± 0.2	4.4 ± 0.4
140[g]	77 ± 15	2.8 ± 0.3	3.1 ± 0.4	5.1 ± 1.0

[a] S_1, as defined in eq 1, denotes the maximum value for soluble protein after precipitation but before total resolubilization. [b] S_2 denotes the soluble species after salting-in defined in eq 1. [c] n = 1, m = 2. [d] n = 1, m = 3. [e] n = 1, m = 3. [f] n = 1, m = 4. [g] n = 1, m = 3.

Table VIII. Cation-Induced Solubility of Bovine Whole Casein at 1 °C

cation	k_1, L/mol	n	k_2, L/mol	m	S_2,[a] g/L
Mg^{2+}	29 ± 1.45	4	4.31 ± 0.23	4	4.4 ± 0.4
Ca^{2+}	19 ± 1.30	3	4.53 ± 1.38	1	7.3 ± 1.2
Co^{2+}	81 ± 8.52	3	2.68 ± 0.59	1	1.7 ± 0.7
Cu^{2+}	1132 ± 205	1	8.71 ± 0.55	4	1.3 ± 0.1

[a] S_2 = concentration of soluble bovine whole casein as defined in eq 1.

Table IX. Cation-Induced Solubility of Caprine Whole Casein at 1 °C

cation	k_1, L/mol	n	k_2, L/mol	m	S_2,[a] g/L
Mg^{2+}	258 ± 101	1	6.6 ± 0.2	6	4.2 ± 0.3
Ca^{2+}	58 ± 6	1	4.6 ± 1.0	3	3.1 ± 0.8
Co^{2+}	1456 ± 38	1	1.6 ± 0.5	3	8.7 ± 1.7
Cu^{2+}	8432 ± 2759	1		1	0.7 ± 0.2

[a] S_2 = concentration of soluble caprine whole casein as defined in eq 1.

at 1 °C where nearly complete salting-in of α_{s1}-A occurred, the variation of solubility with added KCl indicated that the salting-in effect is essentially electrostatic and could result from competition between K$^+$ and Ca^{2+} for binding sites. Here, for whole caseins, only a moderate degree of salting-in occurs, indicating stronger charge interactions, even in β-casein-rich caprine casein at 1 °C. The estimated S_1 values presented in Tables VI and VII reflect the electrostatic contributions of KCl as a result of the binding of K$^+$/Ca^{2+} to negative charged groups on the whole caseins. The lack of total resolubilization reflects a more stable precipitate due to colloid formation and subsequent destabilization by $CaCl_2$.

An inspection of the k_1 values from Tables VI and VII for both caseins shows significantly larger values for caprine whole casein. This effect is consistent with stronger Ca^{2+}– casein interaction with phosphate or other groups having a higher affinity for Ca^{2+} ions in the caprine α_{s1}-casein and whole casein at 1 °C.

D. Effect of Various Cations at 1 °C. The shapes of the solubility profiles of the bovine and caprine whole casein such as those shown in Figure 7 are dictated by both the nature of the cations and the interactions of the protein charged groups with these cations.

Tables VIII and IX show the binding and solubility parameters for the bovine and caprine whole casein derived from the data in Figure 7A for bovine casein and in Figure 7B for caprine casein. Addition of low levels of the divalent salts gave rapid decrease in solubility, indicating that bovine and caprine whole caseins are sensitive to salt-induced aggregation. However, changes in solubility were high depending upon the nature of the cation. In the presence of Co^{2+} and Cu^{2+}, increasing salt concentrations further decreased solubility, suggesting further aggregation of the proteins, whereas with Mg^{2+} and Ca^{2+} a less marked
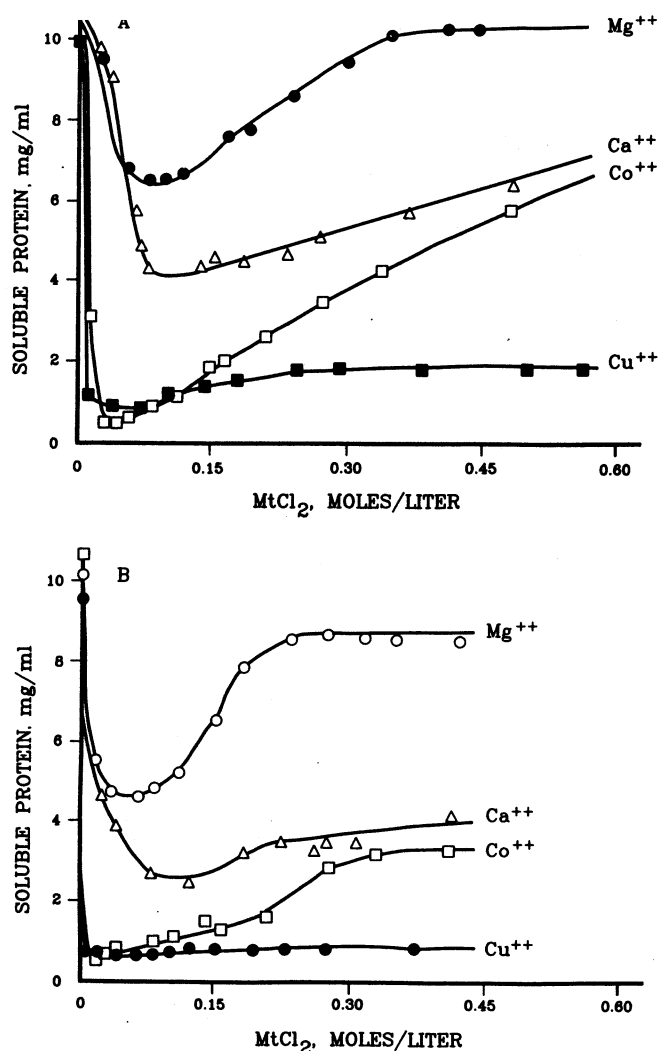
Figure 7. (A) Solubility at 1 °C of bovine whole casein as a function of increasing concentration of various salts. (B) Solubility of caprine whole casein as a function of increasing concentration of various salts. Data in triplicate were fitted by eq 3; curves are as explained in Figure 1. Results are given in Tables VIII and IX.

change in solubility was observed. The decrease in solubility with increasing concentrations of divalent salts was more pronounced for caprine whole casein (Figure 7B) than for bovine whole casein (Figure 7A), which indicates that caprine whole casein is always more sensitive to aggregation in the presence of low levels of these salts. Changes in k_1 are consistent with the above observations (Tables VIII and IX).

The distribution and localization of charged sites on a protein surface can also affect specific ion–protein interactions. The difference between the estimated binding and solubility parameters for bovine and caprine whole casein presented in Tables VIII and IX, respectively, can be related to the distribution of charged residues in the amino acid sequences of the casein molecules. Three-dimensional models for the caseins (Kumosinski et al., 1991a,b) show an exposed negatively charged region exists between residues 30 and 100 of α_{s1}-casein. Such a region of negative charges is not found in bovine β-casein. Increased occurrence of negative amino acid side chains in α_{s1} and its higher ratios in bovine (Table IV) will dictate that at elevated Ca^{2+} concentrations more calcium ion binding may occur. This could result in more soluble material for bovine caseins. At higher concentration of cations, there is further cation binding which leads to the

formation of a protein surface with an apparent net positive charge and again increased solubility of bovine whole caseins.

Differences in the amino acid side chains of κ-, α_{s}-, and β-caseins in caprine whole casein, where the less charged β predominates, could lead to less repulsive charge–charge interactions, and it would explain its lower solubility and more stable precipitate in the presence of divalent salts (Figure 6B) even at 1 °C (Figure 7B).

Concluding Remarks. The thermodynamic linkage approach provides an appropriate treatment for the analysis and comparisons of casein solubility profiles. Here salting-out and salting-in constants are presumed to be linked to calcium binding constants. Our observations can be explained both by the binding of calcium to phosphate and/or carboxylate groups of the protein and by competitive K^+/Ca^{2+} binding at the calcium binding sites. The estimated binding parameters are influenced by the primary structure of the protein as well as the ratios of the various components, as in the present case for bovine and caprine whole caseins. The purified caprine α_{s1}-casein exhibited somewhat smaller derived binding parameters, indicating less calcium binding. In the whole caseins, the reverse was true—caprine whole casein always had a stronger affinity (greater k_1) for calcium and for other cations as well. Our present results with caprine whole casein are consistent with the decreased solubility of these proteins as compared to those of cow's milk. That the whole caseins differ in behavior from estimates drawn from their purified components may be of importance. Historically, caseins are purified in the presence of urea, but urea may alter protein structure and introduce artifacts. Studies such as these point out the necessity of examining purified components as well as the original parent systems. These fundamental differences also point to the importance of the ratios of individual caseins as a determining factor in casein functional properties, a fact not to be overlooked in future breeding and/or genetic engineering projects.

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